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Pathogenesis of *Soybean mosaic virus* in soybean carrying *Rsv1* gene is associated with miRNA and siRNA pathways, and breakdown of AGO1 homeostasis

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ABSTRACT

Profiling small RNAs in soybean Williams 82 (*rsv*), susceptible to *Soybean mosaic virus* (SMV, the genus *Potyvirus*, family *Potyviridae*) strains G2 and G7, and soybean PI96983 (*Rsv1*), resistant to G2 but susceptible to G7, identified the microRNA miR168 that was highly overexpressed only in G7-infected PI96983 showing a lethal systemic hypersensitive response (LSHR). Overexpression of miR168 was in parallel with the high-level expression of *AGO1* mRNA, high-level accumulation of miR168-mediated *AGO1* mRNA cleavage products but with severely repressed AGO1 protein. In contrast, AGO1 mRNA, degradation products and protein remained without significant changes in G2- and G7-infected Williams 82. Moreover, knock-down of *SGS3*, an essential component in RNA silencing, suppressed *AGO1* siRNA, partially recovered repressed AGO1 protein, and alleviated LSHR severity in G7-infected *Rsv1* soybean. These results suggest that both miRNA and siRNA pathways are involved in G7 infection of *Rsv1* soybean, and LSHR is associated with breakdown of AGO1 homeostasis.

Introduction

To protect themselves from viral attack, plants have developed a sophisticated system termed RNA silencing against the invading virus, which represents a primitive, natural immune system of defense (Baulcombe, 1999; Bosher and Labouesse, 2000; Catalanotto et al., 2000; Matzke et al., 2001; Waterhouse et al., 2001). This innate resistance is executed by small interfering RNAs (siRNA)-directed sequence-specific degradation of complementary viral RNAs. Virus-specific siRNAs (vsiRNAs) are generated from viral double-stranded RNA products and secondary RNA structures cleaved by RNase-III ribonuclease Dicer-like (DCL) proteins (Ding and Voinnet, 2007; Molnár et al., 2005; Várallyay et al., 2010). In response, many viruses have evolved a corresponding system to counteract RNA silencing for their own survival through encoding a viral RNA silencing suppressor (VSR), the viral protein that inhibits RNA silencing efficiently *via* either preventing siRNAs generation or inhibiting/interfering the incorporation of siRNAs into the RNA-induced silencing complex (RISC) (Anandalakshmi et al., 1998; Burgyán, 2008; Pumplin and Voinnet, 2013). This reciprocal adaptation and counter-adaptation process results from a co-evolutionary arms race between the host and the virus (Obbard et al., 2009; Zhang et al., 2006).

Another RNA silencing pathway is mediated by microRNAs (miRNAs), a class of non-coding RNAs 20–24 nucleotides in size that regulate gene expression in eukaryotes by translational inhibition or cleavage of complementary mRNAs (Mallory and Bouché, 2008). miRNAs have a pivotal role in a wide variety of biological processes such as maintenance of genome integrity, development, hormone responses and feedback mechanisms as well as biotic and abiotic stress responses (Voinnet, 2009). In addition to regulating the expression of endogenous genes, miRNAs are also indispensable for the innate immune system in animals and plants. For instance, the human cellular miR-32 effectively restricts the accumulation of the retrovirus primate foamy virus type 1 (PFV-1) (Lecellier et al., 2005). miR122 is specifically expressed and highly abundant in the human liver, and

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the sequestration of miR-122 in liver cells results in a marked loss of autonomously replicating hepatitis C viral RNAs (Jopling et al., 2005). In plants, several lines of evidence suggest that virus infections are often associated with the altered levels of endogenous miRNAs and their mRNA targets. The levels of mature miR164, miR164a precursor and its target CUC1 mRNA are increased in *Arabidopsis* plants infected by *Tobacco mosaic virus* Cg (TMV-Cg) or *Oilseed rape mosaic virus* (ORMV) (Bazzini et al., 2009). miRNAs in cotton plants are misregulated by infection with *Cotton leafroll dwarf virus* (CLRDV) and some CLRDV-induced symptoms may be correlated with the deregulation of miRNA and/or epigenetic networks (Romanel et al., 2012). In rice, *Rice stripe virus* (RSV) infection induces the expression of novel phased miRNAs from several conserved miRNA precursors (Du et al., 2011). In *Nicotiana benthamiana* and *Arabidopsis*, the expression of miR168 and AGO1 mRNA is up-regulated in response to infection by several plant viruses (Havelda et al., 2008; Várallyay et al., 2010, 2013; Vaucheret et al., 2006), suggesting both miRNA and siRNA pathways are involved in virus infections. AGO1 protein is a central component of the RISC in the miRNAs/siRNAs-mediated PTGS pathways (Mallory and Vaucheret, 2009). AGO1 has been shown to be responsible for translational inhibition or cleavage of complementary target mRNAs in the miRNA pathway (Mallory and Bouché, 2008; Mallory et al., 2008). Regulation of *AGO1* by miR168 is through a feedback regulatory loop that maintains AGO1 homeostasis by action of miR168 and AGO1-derived siRNAs (Li et al., 2012; Mallory and Vaucheret, 2009; Vaucheret et al., 2004). AGO1 homeostasis is essential for two additional regulatory mechanisms, *i.e.*, transcriptional co-regulation of *MIR168* and *AGO1* genes and posttranscriptional stabilization of miR168 by AGO1 (Li et al., 2012; Vaucheret et al., 2006). Moreover, a recent work described that the enhanced expression of AGO1 mRNA is not accompanied by increased AGO1 protein accumulation in the virus-infected plants, and the p19 RNA-silencing suppressor, a viral protein of tombusviruses mediates the induction of miR168 accumulation and the down-regulation of the endogenous AGO1 protein level (Várallyay et al., 2010, 2013), suggesting that the *AGO1*-miR168 feedback regulation mechanism may play a role in the virus infection process.

Soybean mosaic virus (SMV) is a member of the genus *Potyvirus* in the *Potyviridae* family and its genome is a single-stranded positive-sense RNA. To date, numerous SMV isolates have been classified into seven distinct strains (G1 to G7) based on their differential responses on susceptible and resistant soybean cultivars in North America (Cho and Goodman, 1979). Three independent resistance loci (*Rsv1*, *Rsv3*, and *Rsv4*) with different SMV strain specificities have been identified, and these three loci are all dominant *R* genes that have been mapped to the respective molecular linkage groups F, B2, and D1b (Gore et al., 2002; Gunduz et al., 2002; Hayes et al., 2000; Jeong et al., 2002; Zheng et al., 2005). *Rsv1*, found in soybean cultivar PI96983, confers resistance to the SMV strains G1 to G6 but susceptible to the resistance breaking strain G7 (Chowda-Reddy et al., 2011; Yu et al., 1994). A lethal systemic hypersensitive response (LSHR) is induced by SMV G7 infection in PI96983 carrying *Rsv1*, which is associated with up-regulation of the PR-1 protein gene transcript (Hajimorad and Hill, 2001). To better understand SMV–soybean interactions, global gene expression changes in soybean plants infected by a G2 isolate were monitored during the course of infection using microarray (Babu et al., 2008). A number of genes involved in defense were found to be down-regulated or not affected at the early stages of infection but up-regulated at the late stages, indicating that the plant immune responses are suppressed or not activated until late in the infection. Such a delayed defense response may be critical for SMV to establish its systemic infection (Babu et al., 2008). Consistently, some miRNAs (miR160, miR393 and miR1510) have been shown to be involved in soybean resistance to SMV infection (Yin et al., 2013).

In this study, we show that the specific induction of miR168 accumulation in SMV-infected plants, which was in parallel with an increased *AGO1* mRNA expression. Infection of *Rsv1* soybean by SMV G7 was associated with the highly accumulated *AGO1* mRNA but with low AGO1 protein levels. Moreover, the enhanced accumulation of miR168 spatially overlapped with virus-occupied sectors, which was accompanied with a dramatic increase of miR168-mediated *AGO1* mRNA cleavage products. Furthermore, we show that silencing *Suppressor of Gene Silencing* 3 (*SGS3*) in *Rsv1* plants reduced the level of *AGO1* siRNAs, partially recovered the suppressed level of AGO1 protein resulting from G7 infection, and alleviated LSHR severity. These results suggest that both miRNA and siRNA pathways are involved in pathogenesis of SMV G7 in *Rsv1* soybean through disruption of AGO1 homeostasis.

Results

Identification of soybean miRNAs associated with SMV infection by deep sequencing

To study soybean innate resistance to SMV infection, we inoculated with SMV G2 and G7 strains two soybean cultivars, Williams 82 (*rsv*) carrying no resistance gene and PI96983 (*Rsv1*) carrying the resistance gene *Rsv1*. Consistent with previous publications (Chowda-Reddy et al., 2011; Hajimorad et al., 2003; Hajimorad and Hill, 2001), PI96983 was resistant to G2 but susceptible to G7 by showing a lethal systemic hypersensitive response (LSHR), whereas Williams 82 carrying no resistance gene was susceptible to both G2 and G7 strains (Fig. S1). To investigate miRNAs involved in antiviral response to SMV, six small RNA cDNA libraries from mock- and SMV-infected (G2 and G7) leaves in *rsv* and *Rsv1* soybeans were constructed and subjected to deep sequencing. The small RNA sequences were mapped to the soybean reference genome (Glyma1, Ensembl, http://plants.ensembl.org/Glycine_max/Info/Index) and the SMV genome, and aligned to the known soybean miRNAs database (miRBase 19, http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=gma). For each library, more than 50% of small RNA sequences perfectly matched to the soybean genome (Table S1). Among a large number of miRNAs that were up- or down-regulated in SMV-infected plants, the most strikingly up-regulated miRNA species was miR168 whose expression in the G7-infected *Rsv1* soybeans was up-regulated by more nearly three times ($\log_2$ FC=2.85) in reads *versus* that in the mock-inoculated *Rsv1* soybeans, but no significant difference ($\log_2$ FC=0.22) was found in expression of miR168 in the G2-infected *versus* mock-inoculated *Rsv1* soybeans (Fig. 1A). A high abundance of small RNA reads was found to derive from the region of AGO1 (Glyma16g34300) targeted by miR168 (Fig. 1B). Plant miR168 is one of the most abundant and important miRNAs in plants that regulates *ARGONAUTE1* (*AGO1*) mRNA, a critical component of miRNA/siRNA-mediated posttranscriptional gene silencing (PTGS) pathways (Gazzani et al., 2009; Várallyay et al., 2010; Zhang et al., 2011). Thus, miR168 and its target AGO1 became the focus of this study.

Induction of miR168 accumulation by SMV infection

Northern blot was performed to confirm the induction of miR168 accumulation by SMV infection. A comparative analysis of miR168 detected by northern blotting showed that miR168 accumulation in the G7-infected *Rsv1* plants was about 2.7 times as much as that in the mock-inoculated *Rsv1* plants (Fig. 2A). No significant increment of miR168 was found in G2-inoculated *Rsv1* soybeans that are resistant to G2 (Fig. 2A). miR168 expression in the G2- and G7-infected *rsv* plants was increased by 1.4- and 1.6-

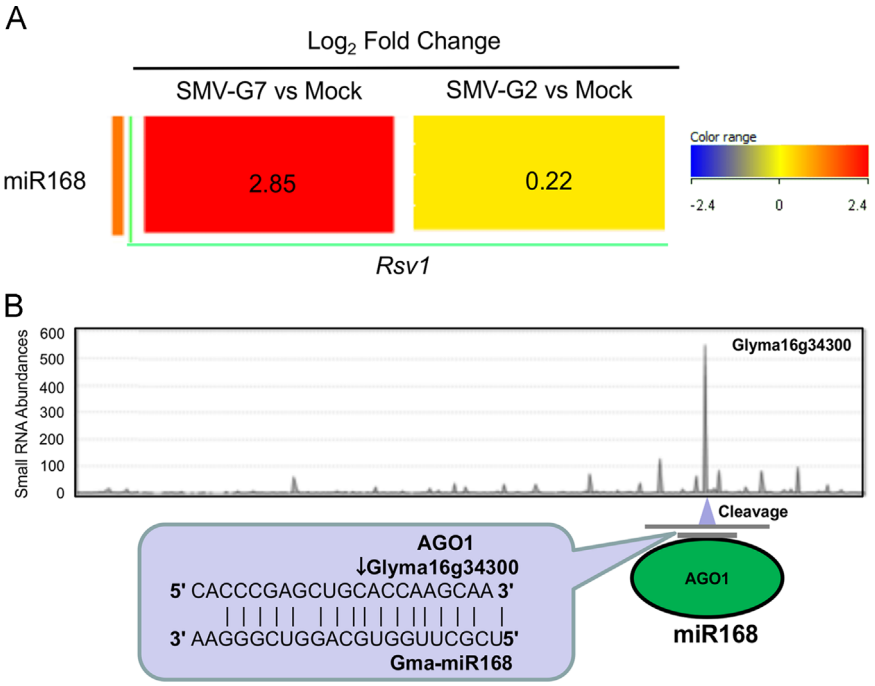

Fig. 1. Identification of miR168 by profiling of small RNAs in SMV-infected soybean plants using deep sequencing. (A) The $\log_2$ fold-change (FC) of miR168 reads in SMV-infected *versus* mock-inoculated plants is indicated. Color range represents the $\log_2$ fold-change value. (B) Alignments of small RNA abundances for the regions of AGO1 (Glyma16g34300) targeted by miR168. A pair-wise alignment of miR168 and its target AGO1 nucleotide sequences is given and the arrow points to the cleavage site. Small RNA abundance normalized in TPM (transcripts per million) is indicated on the *Y*-axis; *X*-axis represents the genomic region of AGO1 targeted by miR168.

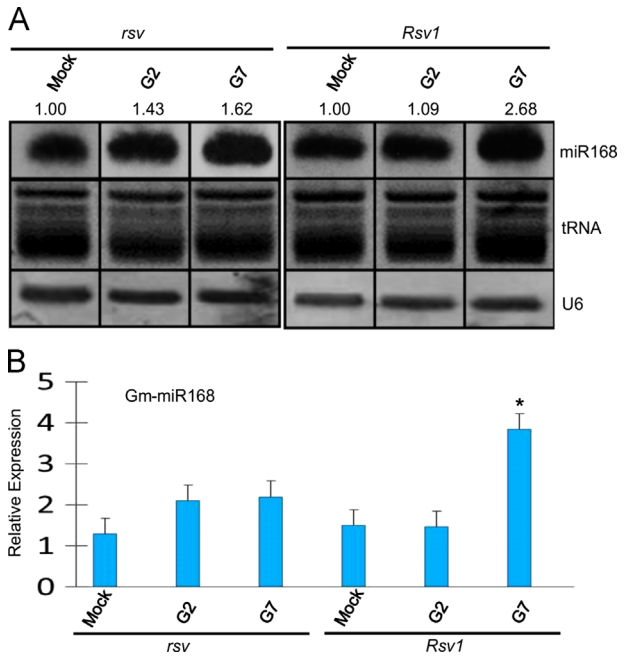

Fig. 2. miR168 accumulation in soybean cultivars Williams 82 (*rsv*) and PI96983 (*Rsv1*) in response to SMV infection. (A) Northern blot detection of miR168 with DIG-labeled RNA probes. Ethidium bromide-stained tRNA was used as a loading control, and hybridized U6 snRNA as an internal control. (B) Comparative analyses of miR168 accumulation in *rsv* and *Rsv1* cultivars inoculated with SMV G2 and G7 strains by Stem-loop RT-qPCR. *rsv*, Williams 82 (carrying no resistance gene) which is susceptible to G2 and G7; *Rsv1*, PI96983 (carrying resistance gene *Rsv1*) which is susceptible to G7 (resistance breaking isolate) but resistant to G2; Mock, inoculated with buffer only; G2, inoculated with SMV G2 strain; G7, inoculated with SMV strain G7; Asterisk, significant difference at $P < 0.01$.

fold, respectively, in comparison with that in the mock-inoculated plants (Fig. 2A). To further validate expression levels of miR168 in the virus-infected plants, stem-loop RT followed by TaqMan quantitative PCR (RT-qPCR) assays (Mestdagh et al., 2008, 2009; Vazquez et al., 2004) was carried out (Fig. 2B). The results were consistent with those obtained from deep sequencing analysis (Fig. 1A and B) and northern blot analysis (Fig. 2A). Therefore, miR168 accumulation is indeed associated with SMV infection.

Enhanced accumulation of miR168 co-localizes with virus-occupied cells

To investigate a spatial relationship between miR168 and SMV in virus-infected plants, young developing leaves with typical SMV symptoms from the G7-infected *Rsv1* plants were fixed and sectioned essentially as described (Hejátko et al., 2006). Consecutive sections were detected for the relative accumulation of SMV viral RNA and miR168 by *in situ* hybridization. Strong miR168 and SMV signals were evident in the tested sections (Fig. 3A). The miR168 signals spatially overlapped only with SMV G7 virus-occupied zones (Fig. 3A). miR4404, as a control, was evenly distributed in the section and no altered positive signals co-localized to miR168 or virus-occupied zones (Fig. 3A), suggesting miR168 is specifically accumulated in the SMV infected cells.

Then, what mechanism led to miR168 accumulation? A recently published work has reported that the increased accumulation of miR164 results from transcriptional activation of the *miR164a* precursor in TMV-cg or ORMV-infected *Arabidopsis* plants (Bazzini et al., 2009). In *Arabidopsis thaliana* plants infected by *Turnip crinkle virus* (TCV), *Ribbgrass mosaic virus* (RMV) or *Cucumber mosaic virus* (CMV), the activation of expression of the *miR168* precursor and increased processing of the precursor have been suggested to be primarily responsible for the increased accumulation of miR168 (Várallyay et al., 2010). To investigate if this was also the case in the G7-infected *Rsv1* plants, we performed northern blot analyses to detect the accumulation of the *miR168* precursor in the mock-inoculated and G7-infected *Rsv1* plants (Fig. 3B). The results show that both stem-loop RNA (~125 nt) and loop intermediates (~70 nt) in the G7-infected leaves were present at much higher

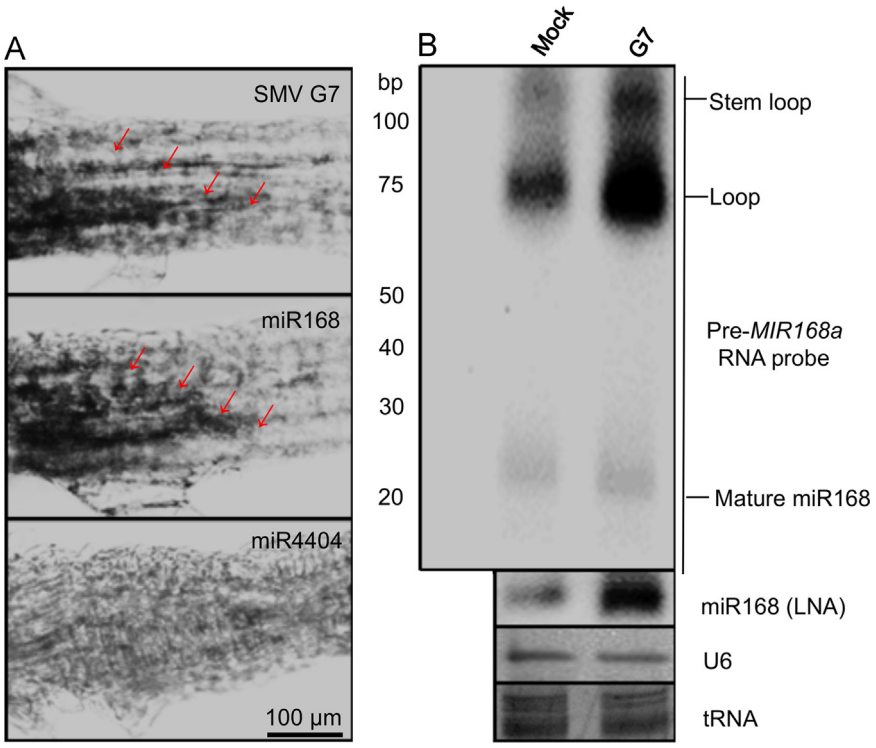

Fig. 3. Detection of miR168 in virus-infected leaves by *in situ* hybridization and Northern blotting. (A) *In situ* detection of SMV G7 viral RNA and miR168 on consecutive sections (10-μm) of systemically infected young leaves from the G7-infected *Rsv1* plants. The upper panel represents the section hybridized with a DIG-labeled RNA probe corresponding to the CP region of SMV G7. The dark spots (some pointed to by red arrowheads) indicate the presence of viral RNA. The middle panel represents the section hybridized with a 5′-DIG labeled locked nucleic acid (LNA) oligonucleotide probe complementary to the mature miR168. The dark spots (some pointed to by red arrowheads) represent the presence of miR168. The low panel shows the section hybridized with DIG-labeled RNA probe complementary to the mature miR4404 as a negative control. Bar, 100 μm. (B) Northern blot detection of miR168 precursors. Northern blotting was performed using a DIG-labeled RNA probe complementary to the *MIR168a* precursor. After stripping, the membrane was re-hybridized with the mature miR168 LNA probe and the U6 snRNA probe, respectively. Ethidium bromide-stained tRNA was used as a loading control.

levels than those in the mock-inoculated leaves (Fig. 3B). Thus, in the G7-infected plants the expression of the stem-loop RNA was highly induced, which was concomitant with the high-level accumulation of the loop intermediate and mature mi168 (Fig. 3B). These data strongly suggest that the transcriptional activation of the *miR168* precursor by SMV infection followed by an efficient processing leads to the elevated level of miR168 in the virus-infected plants.

The elevated level of miR168 is accompanied with high levels of AGO1 mRNA and miR168-mediated AGO1 mRNA cleavage products

It is well known that miR168 has a regulatory role on *AGO1* expression (Havelda et al., 2008; Várallyay et al., 2010, 2013; Zhang et al., 2006). We thus performed northern blotting to detect the expression of *AGO1* mRNA using an *in vitro* transcribed RNA probe corresponding to the *AGO1* mRNA region downstream of the miR168 recognition site. Apparently, the levels of *AGO1* mRNAs or its cleavage products were similar among mock-, G2- and G7-inoculated *rsv* soybean leaves. However, *AGO1* mRNA and its 3′ cleavage products in SMV7-infected *Rsv1* soybeans accumulated to a much higher level than in the mock- or G2-inoculated *Rsv1* leaves (Fig. 4A). Therefore, the increased expression of miR168 and *AGO1* mRNA was in parallel with a dramatic increase of miR168-mediated *AGO1* mRNA cleavage products in the G7-infected *Rsv1* plant (Figs. 3 and 4A).

To confirm the accumulation of the miR168-mediated *AGO1* mRNA cleavage products in G7-infeced *Rsv1* soybean, a recently developed technology, *i.e.*, RNA ligase-mediated 5′ amplification of cDNA ends (RLM-RACE), was employed. This technology has been adapted as a powerful tool for high-throughput analysis of miRNA cleavage products and/or identification of miRNA targets in a genomic perspective (Llave et al., 2011). A 3′ *AGO1* mRNA species of 272 bp in length was found to accumulate at very high levels in the G7-infected *Rsv1* plants compared to the mock-inoculated *Rsv1* plants (Fig. 4C). In contrast, a small amount of this *AGO1* mRNA cleavage species was detected in the G7-infected and mock-inoculated *rsv* plants (Fig. 4C). The identity of the detected mRNA from both samples was confirmed by sequencing. These data suggest that the increased accumulation of miR168 indeed efficiently mediates the cleavage of *AGO1* mRNA in the G7-infected *Rsv1* plants, consistent with the published data that the miR168-mediated cleavage of *AGO1* mRNA is dependent on the level of *AGO1* mRNA (Mallory et al., 2009; Vaucheret et al., 2004).

Translation of AGO1 mRNA is inhibited in the G7-infected Rsv1 soybean

In addition to their essential role in processing target mRNAs, miRNAs have also been implicated in translational repression in plants (Beauclair et al., 2010). As AGO1 protein is responsible for miRNA-guided translational repression (Brodersen et al., 2008), we determined the level of AGO1 protein by western blotting using antibodies against AGO1. It was found that the AGO1 protein level remained almost unchanged in the G2- and G7-infected or mock-inoculated *rsv* plants, similar to what was observed for *AGO1* mRNA (Fig. 4B). In the G7-infected *Rsv1* plants, however, the accumulation of AGO1 protein was severely repressed and only a small amount of AGO1 protein was evident in spite of very high levels of *AGO1* mRNA (Fig. 4B). As the low level of AGO1 protein was coupled with the high level of *AGO1* mRNA and the enhanced accumulation of miR168 (Fig. 2A and B), it is very likely that the

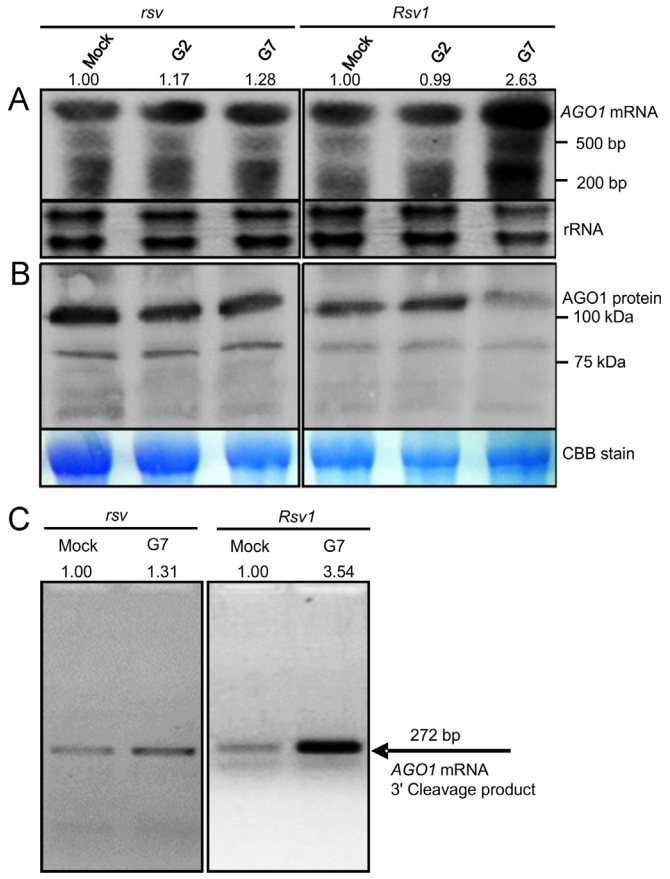

Fig. 4. Accumulation of *AGO1* mRNA and its cleavage products in soybean cultivars Williams 82 (*rsv*) and PI96983 (*Rsv1*) in response to SMV infection. (A) Northern blotting of *AGO1* mRNA in SMV-infected plants. *AGO1* mRNA was detected with an *in vitro* transcribed RNA probe specific for the region downstream of the miR168 recognition site. (B) Western blotting of AGO1 protein with AGO1 polyclonal antibodies. CBB staining of the membrane severs as a loading control. (C) A 3′ miR168 cleavage product (272 nt) of *AGO1* mRNA was detected by RLM-5′ RACE in the mock-inoculated and G7-infected *rsv* and *Rsv1* plant. *rsv*, Williams 82 (carrying no resistance gene) which is susceptible to G2 and G7; *Rsv1*, PI96983 (carrying resistance gene *Rsv1*) which is susceptible to G7 (resistance breaking isolate) but resistant to G2; Mock, inoculated with buffer only; G2, inoculated with SMV G2 strain; G7, inoculated with SMV strain G7.

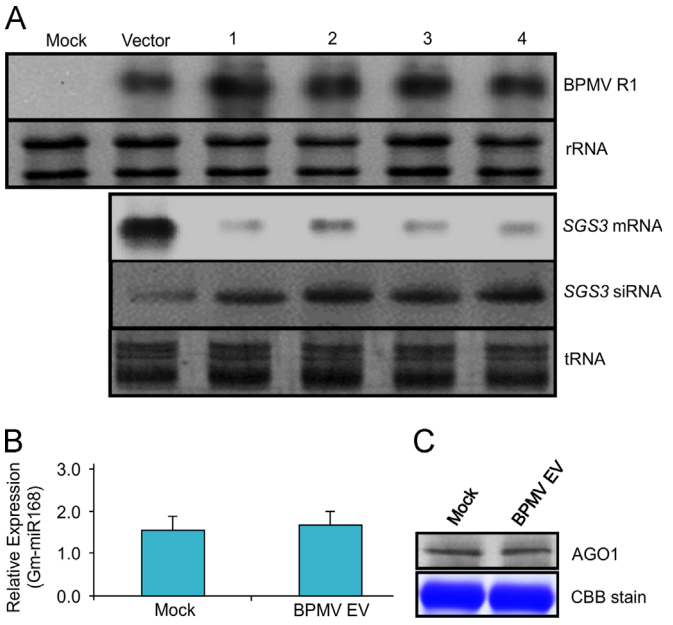

Fig. 5. Efficient silencing of *SGS3* mRNA in soybean *Rsv1* plants by a BPMV-based VIGS Vector. (A) The accumulation of BPMV RNA1, *SGS3* mRNA and *SGS3* siRNA were detected by Northern blot analysis at 14 dpi. Ethidium bromide-stained rRNA and tRNA serve as loading controls. Mock, inoculated with buffer; vector, inoculated with transcripts from the empty vector; four plants (indicated as 1 through 4), inoculated with transcripts derived from the BPMV-based VIGS vector targeting the *SGS3* gene. (B) The accumulation of miR168 detected by Stem-loop RT-qPCR assay in the mock inoculated and BPMV empty vector infected *Rsv1* plants. (C) AGO1 protein detected by Western blotting in the mock-inoculated and BPMV empty vector-infected *Rsv1* plants. CBB staining of the membrane severs as a loading control. Mock, inoculated with buffer only; BPMV EV, inoculated with the BPMV empty vector.

translational inhibition of *AGO1* mRNA in the G7-infected plants is mediated by the high-level miR168 accumulation.

Silencing SGS3 in Rsv1 plants leads to a partial recovery of the diminished AGO1 protein level and an alleviation of LSHR severity induced by G7 infection

To test if the siRNA pathway contributes to the translational inhibition of *AGO1* mRNA, we investigated whether SGS3 is required for the siRNA-mediated degradation of *AGO1* mRNA in G7-infected plants. As an essential component in RNA silencing, SGS3, a plant-specific RNA binding protein, functions in diverse biological processes such as PTGS-mediated antiviral defense and plant development (Mourrain et al., 2000; Peragine et al., 2004; Yoshikawa et al., 2013). SGS3 is required for the production of endogenous *trans-acting* siRNAs (tasiRNAs) and natural antisense transcript-derived siRNAs (natsiRNAs) (Peragine et al., 2004; Yoshikawa et al., 2013). We modified a *Bean pod mottle virus* (BPMV)-based virus-induced gene silencing (VIGS) vector (Kachroo and Ghabrial, 2012) to silence *GmSGS3* gene in soybean. Essentially, the modified BPMV-based VIGS vector contained a DNA fragment proximal to the 3′ end of the open reading frame of *GmSGS3*. The *in vitro* transcripts of the vector were introduced into *Rsv1* plants by mechanical inoculation. Northern blot analysis was performed to monitor the infection of the recombinant BPMV, the level of *SGS3* mRNA, and the production of *SGS3* siRNAs. As expected, the accumulation of *SGS3* mRNA was largely reduced in the BPMV-infected plants with the *SGS3* VIGS construct, which was accompanied with the increased *SGS3* siRNA (Fig. 5A). These data indicate that *SGS3* was silenced efficiently through the production of *SGS3* siRNA. Since *SGS3* was silenced by a viral vector, the accumulation of miR168 and AGO1 expression was also monitored to investigate whether there is an effect on AGO1 homeostasis in the infected plants by the BPMV empty vector. The results from stem-loop RT-qPCR assays and western blotting did not reveal significant difference of miR168 or AGO1 expression in the mock-inoculated and BPMV empty vector-infected plants (Fig. 5B and C).

To investigate whether the accumulation of viral RNA is affected by silencing *SGS3*, we infected both *SGS3*-silenced and non-silenced *Rsv1* plants with G7 and detected the accumulation of positive-stranded and negative-stranded viral RNA. Although both silenced and non-silenced plants developed LSHR after G7 infection, LSHR severity was alleviated in *SGS3*-silenced soybean in comparison with the non-silenced plants (Fig. 6A). Accumulation of positive-stranded viral RNA was slightly reduced in the *SGS3*-silenced soybean as well (Fig. 6B). Silencing *SGS3*, however, significantly inhibited negative-strand viral RNA accumulation (Fig. 6C). We also determined *AGO1* mRNA, *AGO1* siRNAs, and AGO1 protein using RT-qPCR, northern, western blotting, respectively. It was found that the accumulation of *AGO1* mRNA was significantly increased in the *SGS3*-silenced plants compared to the non-silenced plants (Fig. 6D). However, nearly equal levels of *AGO1* mRNA were detected in the *SGS3*-silenced and non-silenced *Rsv1* plants after infection by G7 (Fig. 7A), and silencing *SGS3*

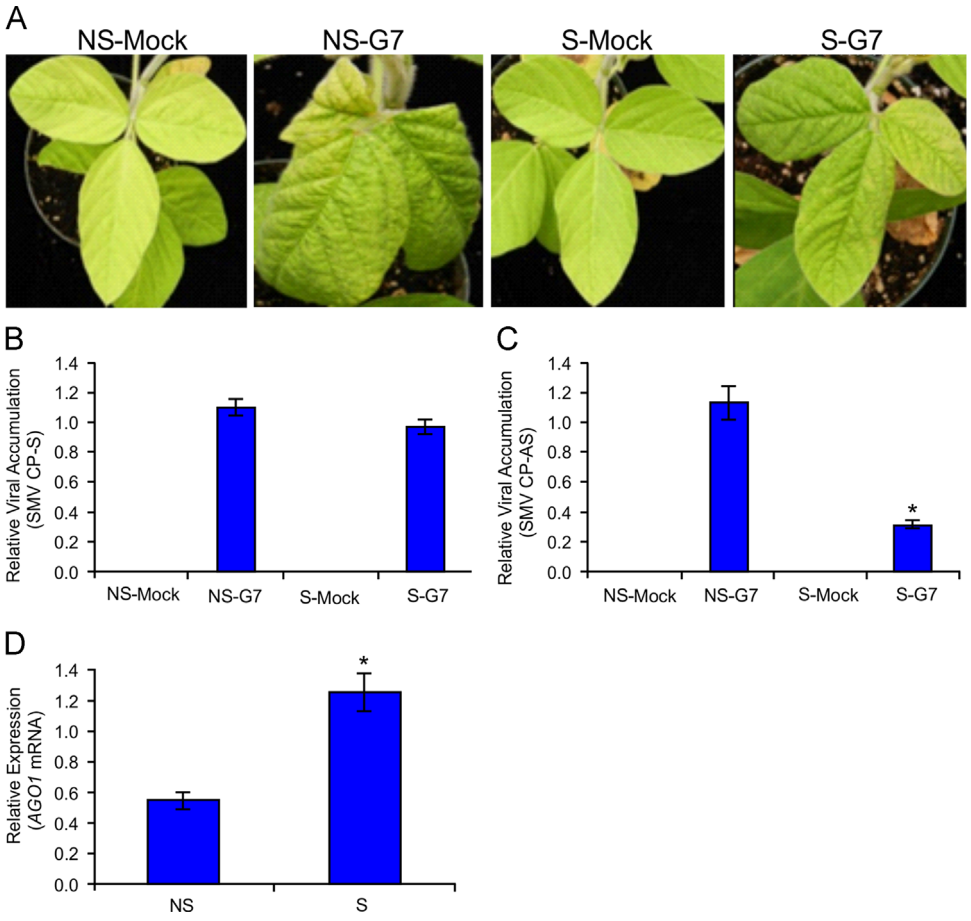

Fig. 6. The effects of silencing *SGS3* on LSHR and viral RNA accumulation. RT-qPCR analyses of accumulated positive-strand and negative-strand viral RNA in the *SGS3*-silenced and control *Rsv1* plants after mock- and G7-inoculation. (A) Symptoms of G7 infection in *SGS3*-silenced or non-silenced *Rsv1* plants. (B) qRT-PCR detection for positive-strand viral RNA with CP-S primers. (C) qRT-PCR detection for negative-strand viral RNA with CP-AS primers. (D) *AGO1* mRNA detection by qRT-PCR assay with specific primers as described in Material and Methods. The experiments were repeated at least three times. S, *SGS3*-silenced *Rsv1* soybean using a modified BPMV virus targeting *SGS3*; NS, non-silenced *Rsv1* soybean pretreated with an empty BPMV vector; G7, inoculation with SMV G7; Mock, inoculation with buffer. *GmElf-1b* severs as an internal control. Asterisk, significant difference at $P < 0.01$.

suppressed the production of *AGO1* siRNAs (Fig. 7B). More interestingly, the inhibited AGO1 protein by G7 infection was recovered partially in the *SGS3*-silenced *Rsv1* plants (Fig. 7C). These results demonstrate that SGS3 is required for the siRNA-mediated *AGO1* mRNA degradation pathway, suggesting that both miRNA and siRNA pathways are required for the maintenance of AGO1 homeostasis in soybean.

Discussion

Viral infection in plants induces the biogenesis of vsiRNAs and endogenous miRNAs that mediate RNA silencing conferring innate resistance to the invading viral pathogen (Havelda et al., 2008; Jones-Rhoades et al., 2006; Várallyay et al., 2010). In a previous study, we suggested that this defense mechanism is responsive to SMV infection (Babu et al., 2008). To screen for miRNAs that may be involved in the defense response, we profiled the miRNA expression in SMV-infected soybeans by deep sequencing. We identified miR168 that was accumulated in SMV-infected *rsv* soybeans and to a much greater extent in SMV-infected *Rsv1* plants (Fig. 1). Upon confirmation of its accumulation by northern blotting and RT-qPCR, we further demonstrated that the accumulation of miR168 resulted from the transcriptional activation and efficient processing of miR168 precursors, which spatially localized to SMV-infected cells (Figs. 2 and 3). miR168 is one of the most highly enriched miRNAs in plants, which regulates *AGO1* expression (Jones-Rhoades et al., 2006; Mallory et al., 2009). AGO1 is the RNA slicer enzyme of miRNA/siRNA pathways and plays a crucial role in various biological processes including antiviral response (Mallory et al., 2009). Therefore, miR168 is involved in SMV G7 infection in *Rsv1* soybean, possibly through regulating *AGO1*.

In this study, we found that AGO1 mRNA and protein were not significantly changed in *rsv* plants infected by SMV (either G2 or G7) (Fig. 4A), suggesting the elevated level of miR168 in these plants (Fig. 2) did not significantly disrupt AGO1 homeostasis. Therefore, AGO1 homeostasis was maintained in both G2- and G7-infected Williams 82 plants. However, G7 infection induced the high-level expression of both miR168 and *AGO1* mRNA in the *Rsv1* soybean (Figs. 2A and 4A). The increased *AGO1* mRNA did not lead to the high level of AGO1 protein, instead AGO1 protein was repressed in the G7-infected *Rsv1* soybean (Fig. 4B). It is very clear that AGO1 homeostasis was disrupted in G7-infected *Rsv1* soybean plants and thus G7 pathogenesis in *Rsv1* soybean is apparently associated with the breakdown of AGO1 homeostasis. Viral infections are often associated with down-regulation of AGO1 to alleviate its anti-viral function. For example, Polerovirus-encoded F box protein (P0) targets the PAZ motif and its adjacent upstream sequence in AGO1 protein and mediates its degradation and destabilization to modulate RNA silencing (Baumberger et al., 2007; Bortolamiol et al., 2007). In the case of tombusviruses, the

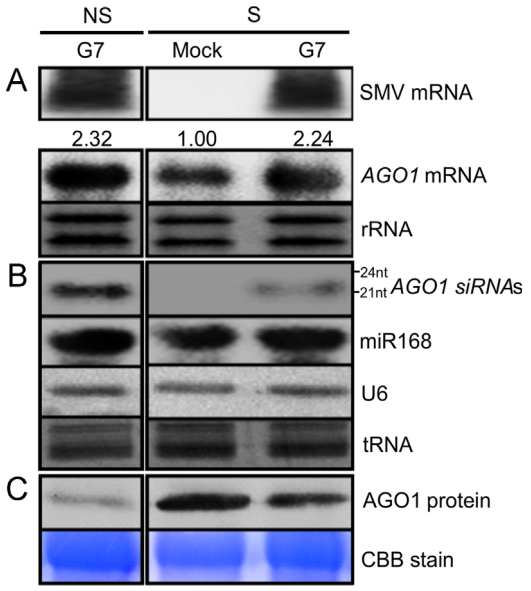

Fig. 7. Recovery of SMV-induced suppression of AGO1 protein by silencing *SGS3* in *Rsv1* soybean. (A) Northern blotting of SMV RNA and *AGO1* mRNA in the non-silenced *Rsv1* plants (NS) and G7-infected *SGS3*-silenced *Rsv1* plants (S). Ethidium bromide-stained rRNA was used as a loading control. (B) Northern blotting of *AGO1* siRNAs accumulation in the non-silenced *Rsv1* plants (NS) and G7-infected *SGS3*-silenced *Rsv1* plants (S). Ethidium bromide-stained tRNA was used as a loading control. (C) Detection of AGO1 protein by Western blotting with AGO1 polyclonal antibodies. CBB staining of the membrane severs as a loading control.

virus-encoded p19 RNA-silencing suppressor mediates the induction of miR168 and down-regulation of the endogenous AGO1 protein level (Várallyay et al., 2010, 2013). The deficiency of AGO1 protein in virus-infected plants can lead to the differential regulation of miRNA targets and eventually result in the development of viral symptoms (Burgyán, 2008). The SMV G7-encoded viral proteins HC-Pro, P3 and CI are virulence determinants on *Rsv1* (Chowda-Reddy et al., 2011). It is yet to be elucidated if these viral proteins or other viral elements are responsible for the induction of miR168 and suppression of AGO1 protein accumulation in the *Rsv1* soybean.

To determine the molecular mechanism by which the translation of *AGO1* mRNA is inhibited, we first checked the accumulation of miR168-mediated *AGO1* mRNA cleavage products. Indeed, the cleavage products of *AGO1* mRNA were remarkably increased in the G7-infected *Rsv1* plants (Fig. 4A and C). Moreover, the *AGO1* mRNA 3′ cleavage product was successfully detected (Fig. 4C) and confirmed by sequencing. Thus, miR168-directed cleavage certainly contributed to the regulation of the *AGO1* mRNA level. Since AGO1 and mature miR168 are associated with polysomes, miR168 may directly mediate the inhibition of *AGO1* mRNA translation, as suggested in several publications (Beauclair et al., 2010; Brodersen et al., 2008; Lanet et al., 2009).

The maintenance of AGO1 homeostasis may also be achieved through the siRNA-mediated *AGO1* mRNA degradation pathway, which requires SGS3, RNA dependent RNA polymerase 6 (RDR6) and silencing defective 5 (SDE5) as well as dicer-like 2 (DCL2) and DCL4 (Mallory and Vaucheret, 2009). In this study, we showed that the *SGS3*-silenced plants accumulated much lower amounts of *AGO1* siRNAs than the non-silenced soybean, and the repressed AGO1 protein by G7 infection was partially recovered in the *SGS3*-silenced plants (Fig. 7B and C), supporting that SGS3 is a crucial component of the siRNA pathway regulating AGO1 homeostasis (Mourrain et al., 2000; Peragine et al., 2004; Yoshikawa et al., 2013). We also found silencing *SGS3* alleviated LSHR severity (Fig. 6A). These data suggest the AGO1-mediated siRNA pathway is required for LSHR and disruption of AGO1 homeostasis is associated with LSHR.

Interestingly, silencing *SGS3* significantly affected the accumulation of G7 negative genomic RNA but only slightly affect the accumulation of G7 positive genomic RNA (Fig. 6B and C). Since both AGO1 and SGS3 are the components of the siRNA pathway, the host defense imposed by the restored AGO1 protein might be traded off by knock-down of *SGS3*. This may explain why silencing *SGS3* did not dramatically affect the accumulation of G7 genomic RNA in *Rsv1* soybean. That silencing *SGS3* led to the suppressed accumulation of G7 negative-stranded RNA was somehow unexpected. It is possible that the partial recovery of AGO1 resulting from silencing *SGS3* may preferentially target G7 negative-stranded RNA rather than the positive viral RNA. The molecular mechanism underlying this observation remains to be understood. In a previous study, the *sgs3* mutants exhibited enhanced susceptibility to CMV but not to *Turnip mosaic virus* (TuMV) or *Turnip vein-clearing virus* (TVCV) (Mourrain et al., 2000), it is not clear if this differential susceptibility is also mediated by AGO1. These data strongly highlight the complex role of the AGO1-mediated pathway in viral infections.

The feedback regulation of *AGO1* mRNA by miR168 is essential for normal plant development; the excess or deficiency of AGO1 protein caused by abiotic or biotic stress interferes with miRNA-guided gene expression, resulting in developmental defects, abnormal phenotypes or symptoms (Mallory et al., 2009). As discussed above, the AGO1 level is kept in check by miR168-directed *AGO1* mRNA cleavage, translational inhibition, siRNA-mediated *AGO1* mRNA degradation, and AGO1-mediated posttranscriptional stabilization of miR168 (Lanet et al., 2009; Mallory and Vaucheret, 2009; Várallyay et al., 2010; Vaucheret et al., 2006, 2004). Thus, the maintenance of AGO1 homeostasis is essentially regulated by coordinated miR168- and siRNA-mediated pathways. Accordingly, we propose a model for the breakdown of AGO1 homeostasis in the G7-infected *Rsv1* soybean (Fig. 8). G7 infection induces the over-expression of *AGO1* mRNA that triggers the *AGO1* siRNA-mediated silencing pathway for the enhanced *AGO1* mRNA degradation. SGS3, RDR6, SDE5 and DCL4/2 are likely involved in this process (Fig. 8). On the other hand, G7 infection also induces the over-accumulation of miR168, which mediates cleavage or translational inhibition of *AGO1* mRNA, leading to repression of the AGO1 protein (Fig. 8). Therefore, these two pathways together break down the equilibrium of AGO1 and down-regulate AGO1. However, since AGO1 may be negatively regulated by ZWILLE/PINHEAD/AGO10 (ZLL) activity (Brodersen et al., 2008), it remains unclear if the excess miR168 may target other *AGO* mRNAs such as *AGO10* to break AGO homeostasis. G7 infection may also induce other miRNAs such as the miR2118/miR482 family to down-regulate the activity of the plant *NB–LRR* defense gene family through the production of phased tasiRNAs (Zhai et al., 2011). The precise mechanism by which AGO1 homeostasis in the *Rsv1* soybean is broken by G7 infection through excess miR168-mediated cleavage of *AGO1* mRNA and translational inhibition pathways remains to be further investigated.

Materials and methods

Soybean cultivars, SMV isolates, viral inoculation and detection

Soybean (*Glycine max*) susceptible cultivar Williams 82 (*rsv*) and resistant cultivar PI96983 (*Rsv1*) were grown in a growth chamber with 16 h of light at 22 °C and 8 h of darkness at 18 °C. The infectious clones of SMV isolate L (a G2 strain) (Gagarinova et al., 2008) and G7 (Hajimorad et al., 2003) were used in this study. SMV infectious clones were initially introduced into Williams 82 (Gagarinova et al., 2008; Hajimorad and Hill, 2001). The resulting SMV-infected leaves were used as an inoculum for

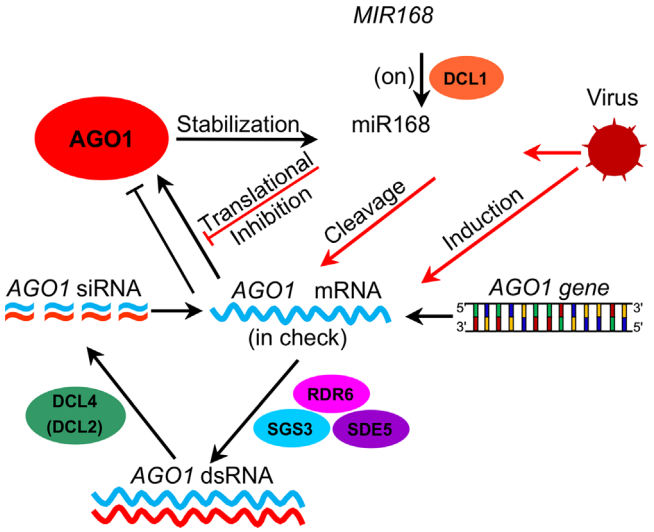

Fig. 8. A proposed model for the breakdown of AGO1 homeostasis by SMV G7 infection through the excess miR168-mediated cleavage and translational inhibition pathways. Normally, AGO1 level can be kept in check by miR168-directed *AGO1* mRNA cleavage and/or translation inhibition, siRNA-mediated *AGO1* mRNA degradation and AGO1-mediated posttranscriptional stabilization of miR168. In host defense response of *Rsv1* plants, G7 infection induces the over-expressed *AGO1* mRNA to trigger the *AGO1* siRNA-mediated the enhanced *AGO1* mRNA degradation pathway, resulting in the reduced AGO1 level, which requires SGS3, RDR6 and SDE5 as well as DCL4/2. At the same time, G7 infection also induces the over-accumulated miR168, which incorporated into AGO1 to mediate the cleavage activity and translational inhibition of *AGO1* mRNA. Eventually, the AGO1 homeostasis was destroyed by G7 infection, resulting in severe viral symptoms and developmental defects. dsRNA, double-stranded RNA; RDR6, RNA Dependent RNA Polymerase 6; SDE5, Silencing Defective 5; SGS3, Suppressor of Gene Silencing 3; siRNA, short interfering RNA.

mechanical inoculations as previously described (Gagarinova et al., 2008; Hajimorad and Hill, 2001).

Virus detection was performed by RT-PCR or RT-qPCR analysis at 15 days post-bombardment or post-inoculation using SMV CP primers SMV-CP-F (5′-TCAGGCAAGGAGAAGGAAGG-3′) and SMV-CP-R (5′-CTGCGGTGGGCCCATGC-3′). The soybean gene *elongation factor 1b* (*GmElf-1b*) was used as an internal control with primers GmElf-1b-F (5′-ACCGAAGAGGGCATCAAATCCC-3′) and GmElf-1b-R (5′-CTCAACTGTCAAGCGTTCCTC-3′). Three independent experiments were performed with five Williams 82 and PI96983 (*Rsv1*) seedlings for each experiment.

For RT-qPCR to quantify positive- or negative-strand SMV genomic RNA, total RNA (1 μg) was reverse transcribed with Super script III Reverse Trancriptase kit (Life) using specific primers (5′-TCTTCTGCAAACGCGGAACC-3′ and 5′-CAAATGAAGGCTGCAGCT-3′) (for positive-strand and negative-strand detection, respectively) according to the manufacturer's instructions. qPCR was performed using the following primer pairs: SMV-CP-S-F (5′-TCAGGCAAGGAGAAGGAAGG-3′) and SMV-CP-S-R (5′-TCTTCTGCAAACGCGGAACC-3′) for positive-strand viral RNA detection, and SMV-CP-AS-F (5′-CAAATGAAGGCTG-CAGCT-3′) and SMV-CP-AS-R (5′-AGCCTTTACTGCTGTGGG-3′) for negative-strand viral RNA detection. The experiments were repeated three times.

Construction of small RNA cDNA library, deep sequencing and bioinformatic analyses

Small RNA cDNA libraries derived from 15 SMV-infected plants were constructed using TruSeq™ small RNA sample prep kit (Illumina) following the manufacturer's instructions. The cDNA libraries were quantified using a library quantification qPCR kit (KAPA Biosystems) on a CFX96 real-time PCR detection system (Bio-Rad) and using a high sensitivity DNA chip on an Agilent 2100 Bioanalyser (Agilent) before loading for deep sequencing in an Illumina Miseq system. After the low quality reads and the adapter sequences were trimmed, the sequences were mapped to the soybean reference genome (Glyma1, Ensembl) and aligned to soybean known miRNAs database (miRBase 19) using the Avadis NGS software (Strand Life Sciences, version 1.4.7) following small RNA analysis pipeline.

BPMV VIGS construction, in vitro transcription and inoculation

For generation of an *SGS3* silencing vector, the BPMV vector used modified essentially as described (Kachroo and Ghabrial, 2012). A 195-bp *SGS3* fragment was amplified with genomic DNA extracted from leaves of the soybean accession PI96983 (*Rsv1*) using forward primer 5′-CGCGGATCCGCGAAGGACAATGGAAGAAA-3′ containing a *Bam*HI site (underlined) and reverse primer 5′-CGCTGCACCCTTGACGGCTTCACCTTCTC-3′ containing an *Eco*RV site (underlined). The PCR product was digested with *Bam*HI and *Eco*RV and cloned into *Bam*HI/*Msc*I-digested pGG7R2-V to generate construct pGG7R2-SGS3. The insert in the construct was verified by sequencing. For inoculation, the constructed plasmids were used as templates for *in vitro* transcription as previously described (Claudia et al., 2011). The resulting transcripts (a mixture with equal amount of RNA1 and RNA2 transcripts) were rub-inoculated to carborundum-dusted unifoliate leaves of soybean cultivar PI96983. Three biological replicates for each treatment consisting of 15 *Rsv1* plants were performed.

RNA isolation and northern blotting

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and small RNA fractions (smaller than 200 nt) were purified from total RNA using a mirVana miRNA isolation kit (Ambion) following the manufacturer's instructions. Northern blot for Gm-miR168 detection was carried out using 5′ digoxin (DIG) labeled locked nucleic acid (LNA) oligonucleotide probes (5DigN/TTCCCGACCTGCACCAAGCGA, Exiqon). Briefly, small RNA fractions were separated on a 15% polyacrylamide (19:1 acrylamide/bisacrylamide) gel with 1 × TBE containing 8 M urea, and then transferred to a positively charged nylon membrane (Roche) in 1 × TBE at 200 mA for 1 h. After blotting, the RNA was cross-linked to the membrane by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) method as previously described (Kim et al., 2010). Pre-hybridization and hybridization were carried out in the UltraHyb Oligo buffer (Ambion) at 50 °C for 1 h and overnight, respectively. After hybridization, the membrane was washed in a low stringency buffer solution (2 × SSC, 0.1% SDS) at 50 °C twice for 5 min, then in a high stringency buffer solution (0.1 × SSC, 0.1% SDS) at 50 °C twice for 15 min. Probe detection was carried out using the DIG Detection Kit (Roche) according to the manufacturer's protocol. The membrane was scanned with a ChemiDoc XRS imaging system (Bio-Rad) or exposed to an X-ray film (Agfa curix). The blot was hybridized with DIG-labeled-U6 small nuclear RNA (snRNA, GACCATTTCTCGATTTGTGCGTGTC) as an internal control to normalize miRNA accumulation.

To detect *AGO1* mRNA, a 131-bp *AGO1* fragment specific for the downstream region from the miR168 recognition site was amplified by RT-PCR with total RNA extracted from leaves of the soybean cultivar 'Williams 82 (*rsv*)' using primers 5′-CGTGGAGGATATGG-CAGTG-3′ and 5′-CGTGGAGGATATGGCAGTG-3′. The PCR product was cloned into pCR4Blunt-TOPO (Invitrogen) to generate construct pCR4-AGO1-mRNA. The insert was verified by sequencing. To detect *SGS3* mRNA and siRNAs, a 416-bp *SGS3* fragment was amplified by RT-PCR using primers 5′-TCCGATGGTTGTCATTCAGA-3′ and 5′-TAA-TACGACTCACTATAGGGCCTCATTCATTTGCCGAAGT-3′ containing the

T7 promoter (underlined). To detect SMV, a 795-bp PCR fragment covering the CP region was amplified with plasmid SMV G7 using primers 5′-TCAGGCAAGGAGAAGGAAGG-3′ and 5′-AATTAACCCTCACTAAAGGGCTGCGGTGGGCCCATGC-3′ containing the T3 promoter (underlined). To detect BPMV R1, the plasmid of BPMV RNA1 (pGHopR1) was digested with *Sal* I and *Not* I to release the fragment containing the engineered T7 promoter and the full-length RNA1 cDNA. The above described constructs/DNA fragments were served as templates to generate RNA probes *via in vitro* transcription using a MAXIscript® T7/T3 Kit (Ambion) with DIG RNA labeling Mix (Roche) following the manufacturer's instructions. For hybridization, total RNA was separated on a formaldehyde-permeated 1.2% agarose gels, and blotted to Hybond-N$^+$ membranes (Amersham Biosciences). The membranes were pre-hybridized and hybridized with the UltraHyb ultrasensitive hybridization buffer (Ambion) according to the manufacturer's instruction. The membranes were washed at 68 °C and the probes were detected as described above.

To detect *AGO1* siRNAs and *Gm-MIR168a* precursor, a 834-bp *AGO1* fragment and a 227-bp fragment of *MIR168a* precursor (*Pre-MIR168a*) were amplified by RT-PCR with primers 5′-TCCTACTCCTCCTCCTGCAA-3′ and 5′-TGCCTGTGAAGTCAGACCAG-3′ and by PCR of the cDNA clone AK244437 with primers 5′-TCGATGTGATGCCATGCGATTGCGTTTG-3′ and 5′-TTACGGGTAGTGGAATCTCGGAGATGAG-3′, respectively. The resulting RT-PCR or PCR products were cloned into pCR4Blunt-TOPO (Invitrogen) to generate construct pCR4-AGO1-siRNA and pCR4-MIR168a. The constructs were used to generate RNA probes *via in vitro* transcription as above described. Hybridization and probe detection were performed essentially as described above. All the experiments described here were repeated at least three times.

In situ hybridization

In situ hybridization of SMV G7-infected young leaf primordia was performed essentially as described (Hejátko et al., 2006; Traas, 2008). A 5′ DIG labeled LNA oligonucleotide probe complementary to the mature miR168 and a DIG-labeled RNA probe corresponding to the CP region of SMV G7 were used to detect miR168 and SMV viral RNA, respectively. A DIG-labeled RNA probe specific for Gm-miR4404 was produced using a mirVana™ miRNA probe construction Kit (Ambio) according to the manufacturer's protocol as an internal control. After hybridization and probe detection, the samples were mounted in 3% agarose gel and were cut into 10-μm consecutive cross sections using a vibratome (Leica, TV 1000S), the sections were observed on a microscope (Zeiss, Axoskop 2). The images were taken by a Nikon digital camera (Nikon, DXM1200) using ACT-1 software (Nikon, version 2.1.2).

Stem-loop RT-qPCR

The stem-loop RT-qPCR assay was carried out as described previously (Mestdagh et al., 2008, 2009). Briefly, the reverse transcription was performed using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol with a stem-loop RT primer (5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCCCG-3′) binds to the 3′ portion of the miR168. The RT product was amplified using the TaqMan® Universal PCR Master Mix (Applied Biosystems) with a miR168-specific forward primer (5′-TATGTTCGCTTGGTGCAGGTC-3′) and the universal reverse primer (5′-GTGCAGGGTCCGAGGT-3′). Soybean 18S rRNA was used as the internal control with a primer pair (5′-GTCCCTGCCCTTTGTACA-3′ and 5′-CACTTCACCGGACCATTC-3′). Each qPCR assay was performed in triplicate.

RLM-5′ RACE

RNA ligase-mediated 5′ amplification of cDNA ends (RLM-5′ RACE) was performed using the FirstChoice RLM-RACE Kit (Ambion) essentially as previously described (German et al., 2008; Llave et al., 2011). Briefly, the poly (A)$^+$ mRNAs were purified from total RNA using the NucleoTrap@ mRNA purification kits (Machery–Nagel) according to the manufacturer's protocol. Approximately 150 ng mRNAs were ligated to 5′ RACE adapter (250 ng, 5′-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3′). The ligated products were purified using a spin-column chromatography (Roche), and then reverse-transcribed into the first-strand cDNA using an antisense gene-specific primer (GSP, 5′-TGATGCAGATCTTTGTTGGGTA-3′), which is located 250 nt downstream of the miR168 cleavage site. PCR amplification was carried out using the 5′ RACE outer primer (5′-GCTGATGGCGATGAATGAACACTG-3′) and GSP primer. After PCR, an aliquot (10 μl) of the 5′ RACE amplification products was analyzed using 1.5% agarose gel electrophoresis in 1 × TAE buffer.

Protein extraction and immunoblotting analysis

Total protein was extracted at 15 dpi using the P-PER® plant protein extraction kit (Pierce) according to the manufacturer's protocol. Protein concentrations were determined using a protein assay kit (Bio-Rad). The immuoblotting analysis was performed as previously described with minor modifications (Mallory et al., 2009). AGO1 primary antibodies against the peptide DYQGRGRGGPSQQGC were made through a special order from GenScript.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.12.034.

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